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Effects of Butterfly Pea Flower (*Clitoria ternatea*) Extract in a Tris Egg Yolk Extender on Saanen Goat Sperm During Freezing, Cold Storage and Incubation

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This study investigated the effects of butterfly pea flower (*Clitoria ternatea*) extract on Saanen goat semen quality during cold storage, cryopreservation, and incubation. Semen was collected from four male Saanen goats (aged 3-4 years) twice a week for five weeks using an artificial vagina. The semen was diluted with a Tris-based extender containing 0%, 0.5%, 1%, and 1.5% concentrations of the extract. After dilution, semen underwent cold storage at 4-5°C and cryopreservation, with quality assessments on days 1, 3, 6, and 9 for cold storage and at 60, 180, and 300 minutes during incubation at 38°C. Evaluations included sperm motility, viability, membrane integrity, acrosome integrity, and kinematic parameters using a light microscope and CASA system. Results indicated significant improvements in sperm quality with butterfly pea flower extract, particularly at the 1.5% concentration. During cold storage, sperm viability increased from 33.10% (control) to 48.46% (1.5% CTE), and motility rose from 50.85% (control) to 68.92% (1% CTE). Kinematic parameters, including progressive motility and curvilinear velocity, improved significantly, especially at 1.5% CTE. Post-thaw semen exhibited enhanced motility and membrane integrity, with the 1.5% CTE group showing the highest values. During incubation, sperm motility was better preserved in CTE-treated groups, with significant improvements in total and progressive motility observed at 60 and 180 minutes. Overall, butterfly pea flower extract significantly enhanced semen quality during storage, cryopreservation, and incubation, suggesting its potential as an effective natural antioxidant for improving semen preservation in artificial insemination programs.

Keywords: Dairy goat, genetics, semen quality, artificial insemination, cryopreservation, antioxidants, kinematics .

INTRODUCTION

The Saanen dairy goat, recognized for its high milk yield, presents a significant opportunity for enhancing dairy production efficiency in Indonesia (Zykova and Chamurliev, 2024). To maximize the genetic potential of Saanen goats and improve reproductive efficiency, artificial insemination (AI) is essential. AI has been widely adopted as a reproductive technology that aims to enhance genetic quality and optimize productivity in dairy goats (Luo et al., 2019). The use of frozen (cryopreserved) and chilled semen in AI allows for flexibility in breeding strategies, with frozen semen preferred for its long-term storage capabilities and chilled semen utilized for shorter storage periods (Gangwar et al., 2023; Gupta et al., 2011; Lemma, 2011). However, both methods encounter challenges in maintaining sperm quality, particularly during cryopreservation and cold storage, where sperm cells are vulnerable to oxidative stress (Meyers, 2012). This oxidative stress can compromise sperm membrane integrity, motility, and viability. To mitigate these issues, it is crucial to formulate semen extenders with protective agents. Recent research has highlighted the potential of incorporating natural antioxidants into semen extenders to combat oxidative damage (Pavuluri et al., 2024). This study focuses on butterfly pea flower (*Clitoria ternatea*) extract, which is rich in anthocyanins and possesses potent

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antioxidant properties, as a promising additive to enhance sperm quality preservation in both frozen-thawed and chilled semen (Aditya et al., 2024; Gew et al., 2024).

Previous studies have demonstrated the positive effects of adding antioxidants to semen extenders in reducing oxidative damage during cryopreservation and cold storage (Toker et al., 2016; Contreras et al., 2020). While both natural and synthetic antioxidants have been evaluated, concerns about synthetic options' safety and potential toxicity have prompted a shift toward exploring natural alternatives. Current semen extenders often fail to effectively protect sperm from oxidative stress, compromising sperm viability, motility, and membrane integrity. This inadequacy highlights the urgent need for improved formulations to better safeguard sperm during storage. In this context, butterfly pea flower extract (CTE: *Clitoria ternatea* Extract), known for its high anthocyanin content and potent antioxidant properties, emerges as a promising candidate (Vidana Gamage et al., 2021; Kim et al., 2024). This study will specifically examine the antioxidant properties of *Clitoria ternatea* extract, focusing on anthocyanins, its primary bioactive compounds. These anthocyanins can scavenge free radicals, including reactive oxygen species (ROS), thereby protecting sperm cells from oxidative damage (Adewoyin et al.,2017). Additionally, they can chelate metal ions, such as iron and copper, which catalyze ROS formation, further reducing oxidative stress. Furthermore, anthocyanins may enhance the activity of antioxidant enzymes within sperm cells, strengthening their overall defense mechanisms. By addressing these mechanisms, this article aims to highlight the potential of *Clitoria ternatea* extract in mitigating the detrimental effects of ROS on spermatozoa during cryopreservation. Despite its recognized antioxidant potential in various biological applications, its efficacy in enhancing semen quality in dairy goats has not been thoroughly investigated. This study aims to fill this gap by examining the effects of butterfly pea flower extract on the quality of Saanen goat semen during cold storage, cryopreservation, and incubation. We hypothesize that incorporating this natural antioxidant will significantly improve sperm motility, viability, and membrane integrity compared to extenders lacking the extract. This study aims to provide novel insights into the application of natural antioxidants in semen extenders, specifically for dairy goats. In contrast to previous research that primarily focused on synthetic antioxidants or only superficially assessed the impact of natural alternatives, this study offers a comprehensive evaluation of the effects of butterfly pea flower extract on critical parameters such as sperm motility, viability, membrane integrity, and sperm kinematics during chilled storage, post-thawing, and incubation.

MATERIALS AND METHODS

***Study design*:** The current study was conducted on four male Saanen goats aged 3 to 4 years, kept in individual cages at the Faculty of Animal Science, Hasanuddin University, Makassar. The goats were maintained under controlled conditions, with a diet consisting of elephant grass and tofu dregs, providing a protein content of 8.66%. Drinking water was available ad libitum. Semen samples were collected twice a week over a period of five weeks using an artificial vagina. The collected semen had a volume ranging from 0.5 to 1.0 mL, with a concentration of $\geq 3\times10^9$ sperms/mL. The motility of the sperms was ≥80%, and morphological abnormalities were ≤10%. After collection, the semen samples were incubated at 37°C and then evaluated both macroscopically and microscopically to assess their quality.

***Extraction of butterfly pea flower* (*Clitoria ternatea* L.):** The butterfly pea flower (*Clitoria ternatea L.*) was first cleaned under running water. The subsequent drying process involved air-drying followed by dehydration until a dryness level of 90% was achieved, after which the flowers were blended into a fine powder. The extraction of the butterfly pea flower was conducted using the maceration method with 96% ethanol. Specifically, 1 kg of the powdered flower was immersed in 5 liters of 96% ethanol for 3x24 hours, with occasional stirring and replacement of the solvent every 24 hours. The resulting extract was then filtered and subjected to vacuum evaporation at 40°C to obtain a thick extract (Harahap et al., 2023).

***Preparation of extender and processing of semen*:** The diluent used was from Germany. It consisted of 2.42 g of tris amino methane, 1.28 g of citric acid, 2.16 g of fructose, 20% egg yolk, and 6% glycerol. The diluent was enriched with butterfly pea flower extract (CTE) for the treatment groups at concentrations of 0.5%, 1%, and 1.5%, while the control group contained the Tris solution without CTE. Fresh semen from Saanen goats was divided into four groups according to the CTE concentrations (0%, 0.5%, 1%, and 1.5%). Each group had 1 mL stored at 4-5°C for cold storage, with quality assessments focusing on motility and kinematics conducted every 24 hours. The remaining semen was reserved for cryopreservation, following the procedure by Bozkurt et al. (2014). This involved equilibration at 3-5°C for 2-3 hours, pre-freezing at 10 cm above liquid nitrogen for 10-15 minutes, and storage in liquid nitrogen at -196°C as described by Saha et al. (2022). For incubation, semen samples were incubated by thawing them at 38°C for 30 seconds, transferring them to 2 mL tubes, and keeping them in an oven at the same temperature. Sperm kinematics and motility were evaluated using Computer Assisted Semen Analysis (CASA) at intervals of 60, 180, and 300 minutes, based on *Swami* et al. (2017)**.** The thawing process involved taking the straw from the liquid nitrogen container and thawing it in a thawing device for 30 seconds at 37°C. After thawing, the straw was

cleaned and cut near the cotton-plugged end (Diansyah et al., 2023; Gungor et al., 2024).

Parameters and Evaluation of Semen

***Motility and kinematics*:** The evaluation of semen was conducted by pipetting10 µl of thawed spermatozoa, which was placed on a glass slide and covered with cover glass. The sample was observed under a microscope at 400× magnification and then analyzed using a CASA system with the Sperm Vision software, version 3.7.5 (Schubert et al., 2019; Diansyah et al., 2022).

***Viability*:** The viability of spermatozoa was evaluated using the eosin-nigrosin staining method. A drop of thawed semen was mixed with an equal volume of eosin-nigrosin solution on a glass slide and then air-dried. The percentages of live and dead sperm were determined by counting 200 sperm cells under a light microscope at 400× magnification (Mortimer, 2020).

***Membrane Integrity*:** The assessment of sperm plasma membrane integrity was conducted using the hypoosmotic swelling test. A 100 µL aliquot of thawed semen was mixed with 1 mL of hypoosmotic solution (150 mOsm/L) and incubated at 37°C for 60 minutes. After incubation, the percentage of sperm with intact membranes was determined by counting 200 sperm under a phase-contrast microscope at 400× magnification (Ramu and Jeyendran, 2013; Diansyah et al., 2021).

***Acrosome Integrity*:** Evaluation of acrosomal integrity in spermatozoa is indicated by the thick black coloration at the sperm head's tip when semen is exposed to a physiological NaCl solution containing 1% formalin. The evaluation was performed on a minimum of 200 spermatozoa using a light microscope at 400× magnification (Egeberg et al.,2018).

***Abnormality*:** Evaluation of morphological abnormalities of the sperms, including head, midpiece, and tail defects, were determined by preparing a smear of the thawed semen, staining with eosin-nigrosin, and counting 200 sperm cells under a light microscope at 400× magnification (Raj et al., 2021).

***Data Analysis*:** The collected data were analyzed using SPSS software (version 29 IBM) with one-way ANOVA, followed by Duncan's Multiple Range Test (DMRT) when the value was significant ($p < 0.05$). All data are presented as mean ± standard error (SE).

RESULTS

***Evaluation of Saanen Goat Fresh Semen*:** Based on the data presented in Table 1, the evaluation of fresh semen quality from Saanen goats supplemented with butterfly pea flower extract at various concentrations (0%, 0.5%, 1%, and 1.5%) revealed no significant effects on the measured semen parameters. Across all concentrations tested, key indicators such as viability, motility, membrane integrity, and acrosome integrity remained consistent with those observed in the control group, indicating that adding the extract did not adversely affect sperm quality. Although a slight increase in sperm abnormalities was observed at the highest concentration (1.5%), this variation was not statistically significant and did not impact on the overall semen quality. These findings suggest that including butterfly pea flower extract in semen extenders is safe for use at the tested concentrations without inducing detrimental effects on sperm quality. Therefore, this extract may be further utilized in subsequent stages, such as cold storage and cryopreservation, to assess its potential benefits under prolonged storage conditions.

Post-Thawed Semen Analysis: The effects of varying concentrations of CTE on several semen quality parameters of Saanen goats, including viability, motility, membrane integrity, acrosome integrity, and abnormality are presented in Table 2. The data show an overall trend of improved semen quality with increasing CTE concentrations, as evidenced by higher motility, membrane integrity, and acrosome integrity,

Table 1. Effect of *Clitoria ternatea* extract on fresh extended sperm.

CTE (%)	Viability	Motility	HOST	Intact Acrosome	Abnormality
0	85.78±4.67	81.28±0.49	81.92±0.46	83.54±3.02	14.29±0.37
0.5	86.07±4.64	82.72±3.43	82.20±1.02	82.68±3.06	14.71±0.37
1	84.36±4.67	81.52±0.85	81.90±0.56	81.15±0.61	14.57±0.89
1.5	83.99±4.71	80.86±0.44	81.58±0.87	80.63±0.33	14.34±0.50

CTE: *Clitoria ternatea* extract

Table 2. Effect of *Clitoria ternatea* extract on post thawed semen.

CTE (%)	Viability	Motility	HOST	Intact acrosome	Abnormality
0	$33.10±1.50^{a}$	$50.85 ± 1.30^{a}$	$39.33±2.51^{a}$	$40.73±3.75^{a}$	14.72±0.35
0.5	$35.63±1.15^{ab}$	$58.05 ± 1.44^{b}$	$45.26±5.05^{b}$	$47.19±4.66^{bc}$	15.26±0.33
1	$42.85±5.30^{bc}$	$68.92 ± 2.22^{c}$	$48.31±2.87^{bc}$	$49.09±3.06^{bc}$	15.08±0.85
1.5	$48.46±4.77^{b}$	$64.71 ± 1.20^{b}$	$53.74±0.38^{c}$	$54.59±0.93^{c}$	14.86±0.44

CTE: *Clitoria ternatea* extract

^{ab}Different superscripts in columns indicate significant difference ($P < 0.05$).

Table 3. Effect of *Clitoria ternatea* extract on the kinematics of post thawed semen.

	CTE (%)			
PARAMETER	**0**	**0.5**	**1**	**1.5**
PM	33.55 ± 2.35[a]	35.84 ± 3.79[a]	47.21 ± 2.70[b]	49.02 ± 3.25[b]
LM	17.30 ± 2.71[bc]	22.20 ± 3.93[b]	29.70 ± 1.63[a]	15.70 ± 1.47[c]
DCL	29.45 ± 3.43	28.33 ± 2.91	27.35 ± 3.64	29.23 ± 1.46
DAP	17.40 ± 1.05	17.07 ± 0.97	16.47 ± 1.01	17.74 ± 1.64
DSL	13.17 ± 0.58	12.82 ± 0.82	12.13 ± 1.01	12.58 ± 1.09
VCL	73.70 ± 1.92[a]	69.70 ± 1.75[bc]	68.02 ± 1.00[c]	71.84 ± 1.54[ab]
VAP	43.77 ± 1.89	42.56 ± 0.97	41.60 ± 0.82	43.79 ± 1.29
VSL	33.20 ± 0.68[a]	32.34 ± 0.57[a]	31.02 ± 1.38[b]	31.00 ± 0.54[b]
LIN	0.45 ± 1.36	0.46 ± 0.86	0.46 ± 0.83	0.43 ± 1.11
STR	0.76 ± 0.62	0.76 ± 0.99	0.75 ± 1.32	0.71 ± 0.97
WOB	0.59 ± 1.34	0.61 ± 1.43	0.61 ± 1.75	0.61 ± 1.45

CTE: *Clitoria ternatea* extract

[ab]Different superscripts in columns indicate significant difference (P < 0.05).

PM: progressive motility; LM: local motility; DAP: average path distance; DSL: straight linear distance; DCL: curvilinear distance, VAP: average path velocity; VSL: straight linear velocity; VCL: curvilinear velocity; STR: straightness; LIN: linearity.

while sperm abnormality remains relatively consistent across treatments.

The kinematic analysis of post-thaw sperm motility in Saanen goat semen treated with varying concentrations of CTE are shown in Table 3. Post-thaw kinematic evaluation is essential to assess the functional capacity of sperm after the cryopreservation process, as freezing and thawing can significantly affect motility and related parameters. The data shows a general trend of improved kinematic performance at higher concentrations of CTE (1% and 1.5%), suggesting that CTE supplementation may enhance sperm resilience and motility after thawing. These findings provide a basis for further discussion on the potential mechanisms and applications of CTE in improving the quality of cryopreserved semen.

***Kinematics of Saanen Goat sperm upon cold preservation*:** Understanding the kinematic parameters of chilled semen over several days, as presented in Table 4, it is essential for evaluating the functional capability of sperm during extended storage. (Fernandez-Novo *et al.*, 2021).

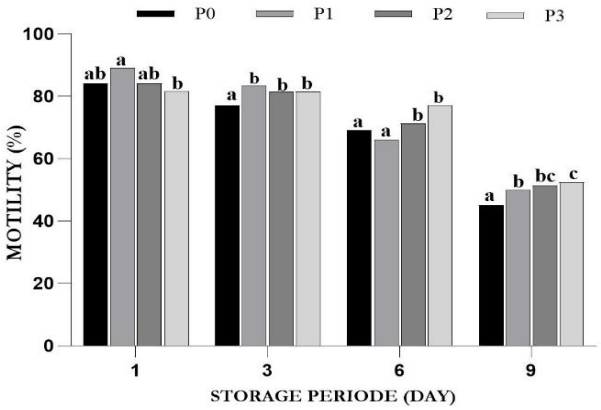

Figure 1. The effects of different CTE concentrations (0%, 0.5%, 1%, and 1.5%) on Total motility (TM) of sperm. Bars represent mean ± SEM. Different superscript letters above bars indicate statistically significant differences between groups (p < 0.05).

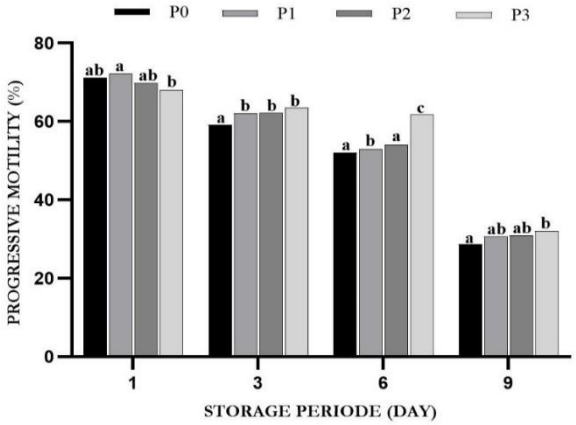

Figure 2. The effects of different CTE concentrations (0%, 0.5%, 1%, and 1.5%) on progressive motility (PM) of sperm. Bars represent mean ± SEM. Different superscript letters above bars indicate statistically significant differences between groups (p < 0.05).

Chilled storage, while commonly used to preserve semen for artificial insemination, often leads to a decline in motility and velocity parameters due to oxidative stress and membrane damage. The addition CTE has been proposed as a protective agent due to its antioxidant properties. In this study, the results indicate that higher concentrations of CTE (1% and 1.5%) were effective in maintaining key kinematic

Table 4. Effect of *Clitoria ternatea* Extract on the Kinematics of Cold Preservation.

PARAMETER	CTE (%)	1 DAY	3 DAYS	6 DAYS	9 DAYS
DCL	0	49.62 ± 1.82^{a}	47.50 ± 1.85^{a}	37.67 ± 2.23^{ab}	29.99 ± 2.111^{a}
	0.5	47.59 ± 1.38a^{b}	40.44 ± 1.88^{a}	39.53 ± 0.79^{a}	31.16 ± 3.794^{a}
	1	46.62 ± 1.40^{b}	45.07 ± 1.58^{ab}	40.62 ± 0.74^{a}	34.91 ± 0.155^{b}
	1.5	44.05 ± 0.73^{c}	44.20 ± 1.62^{b}	35.91 ± 2.20^{b}	37.09 ± 1.969^{c}
DAP	0	25.51 ± 1.07	24.82 ± 1.88	21.36 ± 2.21	18.18 ± 0.386^{ab}
	0.5	25.18 ± 0.66	22.44 ± 1.81	21.08 ± 0.57	17.11 ± 1.444^{b}
	1	24.35 ± 1.25	23.79 ± 1.70	21.77 ± 0.95	19.14 ± 0.660^{a}
	1.5	23.26 ± 1.88	22.82 ± 1.21	19.39 ± 1.32	19.35 ± 1.589^{a}
DSL	0	15.81 ± 1.41	13.88 ± 1.11	14.18 ± 2.98	12.78 ± 0.729
	0.5	15.07 ± 1.41	13.66 ± 1.26	13.84 ± 2.59	11.73 ± 1.006
	1	14.92 ± 1.97	14.68 ± 0.91	14.45 ± 1.36	12.89 ± 0.231
	1.5	13.95 ± 0.68	14.23 ± 1.45	13.09 ± 2.16	12.65 ± 1.987
VCL	0	119.20 ± 1.04^{a}	108.10 ± 0.69^{a}	90.39 ± 2.32^{b}	71.97 ± 2.651^{a}
	0.5	115.90 ± 1.79^{b}	96.07 ± 0.60^{c}	97.17 ± 1.86^{a}	71.93 ± 7.635^{a}
	1	110.60 ± 0.83^{c}	106.60 ± 1.40^{ab}	96.44 ± 1.11^{a}	84.63 ± 1.547^{b}
	1.5	105.50 ± 1.72^{d}	105.50 ± 1.2^{1b}	86.73 ± 1.02^{c}	87.72 ± 4.908^{b}
VAP	0	61.67 ± 1.98^{a}	56.80 ± 1.88^{a}	51.68 ± 1.41^{a}	44.18 ± 0.317^{b}
	0.5	61.75 ± 1.08^{a}	53.44 ± 1.70^{b}	52.29 ± 1.92^{a}	40.06 ± 4.688^{a}
	1	58.26 ± 1.63^{b}	56.77 ± 1.03^{a}	51.95 ± 2.11^{a}	46.71 ± 0.986^{b}
	1.5	56.19 ± 0.84^{b}	54.99 ± 0.83^{b}	46.88 ± 0.75^{b}	46.39 ± 4.390^{b}
VSL	0	38.48 ± 1.79^{a}	32.06 ± 1.31^{c}	34.69 ± 0.96	31.45 ± 0.710
	0.5	37.18 ± 0.90^{a}	32.81 ± 1.50^{c}	34.55 ± 0.67	27.79 ± 3.970
	1	36.10 ± 1.38^{b}	36.32 ± 1.34^{a}	34.70 ± 2.42	31.65 ± 0.175
	1.5	34.05 ± 1.45^{b}	34.65 ± 0.82^{ab}	31.94 ± 2.07	30.59 ± 4.984
LIN	0	0.32 ± 0.55	0.30 ± 1.61	0.38 ± 0.99	0.44 ± 0.006^{a}
	0.5	0.32 ± 1.75	0.34 ± 0.75	0.36 ± 1.08	0.39 ± 0.067^{ab}
	1	0.33 ± 1.88	0.33 ± 0.78	0.36 ± 2.08	0.37 ± 0.005^{ab}
	1.5	0.32 ± 1.07	0.33 ± 0.65	0.37 ± 1.82	0.35 ± 0.037^{b}
STR	0	0.62 ± 1.39	0.56 ± 1.0	0.67 ± 1.14	0.71 ± 0.021
	0.5	0.60 ± 0.53	0.61 ± 0.83	0.66 ± 1.45	0.69 ± 0.064
	1	0.62 ± 1.24	0.62 ± 1.7	0.67 ± 1.27	0.68 ± 0.018
	1.5	0.61 ± 1.08	0.63 ± 1.73	0.68 ± 1.84	0.66 ± 0.045
WOB	0	0.52 ± 1.64	0.53 ± 0.54	0.57 ± 1.31	0.61 ± 0.027^{a}
	0.5	0.53 ± 0.84	0.56 ± 1.75	0.54 ± 1.83	0.56 ± 0.058^{b}
	1	0.53 ± 1.68	0.53 ± 1.42	0.54 ± 0.90	0.55 ± 0.028^{bc}
	1.5	0.53 ± 1.25	0.52 ± 0.87	0.55 ± 2.27	0.53 ± 0.021^{c}

CTE: *Clitoria ternatea* extract

^{ab}Different superscripts in columns indicate significant difference ($P < 0.05$).

DAP: average path distance; DSL: straight linear distance; DCL: curvilinear distance VAP: average path velocity; VSL: straight linear velocity; VCL: curvilinear velocity; STR: straightness; LIN: linearity; WOB: Wobble

parameters, such as progressive motility, curvilinear velocity (VCL), and straight-line velocity (VSL), during storage periods of up to 9 days.

The data on motility presented in Figure 1 indicates a gradual decline in spermatozoa motility during cold storage. On day 1, motility remained high (81%-89%), with P1 being the highest. By day 3, there was a moderate decrease (77%-83%), while P3 began stabilizing. A significant decline was observed on day 6, with P0 dropping to 69%, whereas P3 remained at 77%. On day 9, motility sharply decreased, with P0 at only 47% and P3 being the highest at 52%. Overall, P3 (1.5%) was the most effective in maintaining motility. The data on progressive motility shown in Figure 2 indicates that on day 1, progressive motility values were still high and consistent (68.12%-72.15%), with no significant differences between groups. However, by day 6, P3 maintained the highest value (61.76%), while P0 decreased to 52.03%, and P1 experienced a sharp decline (45.6%). On day 9, a drastic reduction occurred, with P3 remaining the highest at 31.55%, whereas P0 recorded the lowest value (28.72%). Overall, the effectiveness of P3 (1.5%) became evident on day 6 and was more pronounced by day 9.

Table 5. Effect of *Clitoria ternatea* Extract on the Kinematics of Semen Incubated at 38°C After Thawing.

PARAMETER	CTE (%)	60 Min	180 Min	300 Min
TM	0	48.52 ± 1.76[a]	49.17 ± 3.89[b]	30.52 ± 3.64[a]
	0.5	54.18 ± 2.01[b]	43.65 ± 2.08[c]	26.83 ± 3.68[a]
	1	65.44 ± 3.60[c]	50.77 ± 1.81[b]	28.53 ± 3.93[a]
	1.5	63.33 ± 3.46[c]	65.14 ± 2.38[a]	48.40 ± 3.520[b]
PM	0	31.94 ± 2.59[a]	27.98 ± 1.85[a]	11.80 ± 1.40[a]
	0.5	38.55 ± 1.13[b]	27.09 ± 1.87[a]	15.27 ± 3.83[a]
	1	47.65 ± 2.42[c]	34.29 ± 3.07[b]	14.66 ± 2.09[a]
	1.5	46.38 ± 3.46[c]	42.75 ± 1.53[c]	30.69 ± 2.21[b]
LM	0	16.60 ± 1.05	21.20 ± 1.80[a]	18.70 ± 3.60[a]
	0.5	15.60 ± 1.77	16.60 ± 2.93[b]	11.60 ± 1.36[b]
	1	17.80 ± 1.61	16.50 ± 2.73[b]	13.90 ± 3.42[ab]
	1.5	16.90 ± 2.58	22.40 ± 2.35[a]	17.70 ± 2.01[a]
DCL	0	28.80 ± 1.51[a]	25.02 ± 2.71[a]	20.15 ± 1.12[a]
	0.5	33.46 ± 3.25[b]	25.54 ± 3.64[a]	20.08 ± 1.46[a]
	1	31.39 ± 1.92[ab]	27.76 ± 2.05[ab]	19.86 ± 1.09[a]
	1.5	31.57 ± 3.15[ab]	32.43 ± 1.64[b]	26.78 ± 2.98[b]
DAP	0	17.47 ± 1.44	15.33 ± 1.26[a]	13.11 ± 0.82[a]
	0.5	19.65 ± 1.61	16.18 ± 0.51[a]	13.34 ± 0.76[a]
	1	18.43 ± 1.0	16.86 ± 1.39[a]	13.86 ± 1.15[a]
	1.5	17.40 ± 1.94	19.30 ± 1.27[b]	16.02 ± 1.70[b]
DSL	0	13.32 ± 0.55[a]	11.62 ± 0.94	10.44 ± 1.35[a]
	0.5	14.36 ± 0.95[b]	12.66 ± 1.93	10.63 ± 0.76[a]
	1	13.28 ± 1.68a[b]	12.63 ± 1.43	11.20 ± 1.20[ab]
	1.5	12.17 ± 0.57[b]	13.92 ± 1.14	12.98 ± 1.66[b]
VCL	0	70.95 ± 1.15[a]	62.18 ± 1.31[a]	50.65 ± 1.51[a]
	0.5	81.21 ± 1.21[bc]	64.24 ± 1.74[a]	51.48 ± 0.52[a]
	1	78.48 ± 1.75[b]	70.86 ± 1.83[b]	50.37 ± 1.06[a]
	1.5	78.23 ± 1.17[c]	80.25 ± 0.64[c]	64.28 ± 0.88[b]
VAP	0	43.47 ± 0.59[a]	38.95 ± 1.86[a]	33.42 ± 1.21[a]
	0.5	48.33 ± 0.52[b]	40.96 ± 0.97[ab]	34.81 ± 0.59[a]
	1	46.77 ± 1.07[b]	43.08 ± 1.48[b]	36.94 ± 1.06[b]
	1.5	43.50 ± 1.54[a]	48.21 ± 1.30[c]	38.78 ± 1.59[b]
VSL	0	33.27 ± 1.69[a]	29.66 ± 1.46[a]	26.91 ± 0.87[a]
	0.5	35.86 ± 1.20[b]	32.33 ± 0.63[b]	28.15 ± 1.57[ab]
	1	34.02 ± 0.60[b]	32.57 ± 0.66[bc]	29.30 ± 1.34[c]
	1.5	30.63 ± 1.53[a]	34.96 ± 1.75[c]	31.50 ± 0.72[c]
LIN	0	0.47 ± 0.78	0.48 ± 1.22	0.53 ± 1.32
	0.5	0.44 ± 0.60	0.50 ± 1.73	0.55 ± 1.11
	1	0.43 ± 1.51	0.46 ± 1.63	0.58 ± 1.06
	1.5	0.39 ± 1.33	0.44 ± 1.69	0.49 ± 1.74
STR	0	0.77 ± 1.07	0.76 ± 1.54	0.81 ± 0.8
	0.5	0.74 ± 0.94	0.79 ± 1.26	0.81 ± 1.93
	1	0.73 ± 1.17	0.76 ± 1.58	0.82 ± 0.99
	1.5	0.70 ± 1.46	0.73 ± 0.88	0.81 ± 1.06
WOB	0	0.61 ± 1.01	0.63 ± 0.87	0.66 ± 1.52
	0.5	0.60 ± 1.23	0.64 ± 1.44	0.68 ± 0.57
	1	0.60 ± 1.89	0.61 ± 0.95	0.71 ± 0.97
	1.5	0.56 ± 1.71	0.60 ± 1.96	0.60 ± 0.72

CTE: *Clitoria ternatea* extract. [ab]Different superscripts in columns indicate significant difference ($P < 0.05$). TM: total motility; PM:progressive motility; LM: local motility; DAP: average path distance; DSL: straight linear distance; DCL: curvilinear distance VAP: average path velocity; VSL: straight linear velocity; VCL: curvilinear velocity; STR: straightness; LIN: linearity

***Kinematics of Saanen Goat sperm upon Incubation at 38°C*:** Post-thaw incubation is a critical phase to assess the resilience and functional capacity of sperm under physiological conditions, as it simulates the environment during fertilization (Botta et al., 2019).The kinematic parameters of thawed Saanen goat semen incubated at various time points (60, 180,

and 300 minutes) following treatment with different concentrations of CTE as presented in Table 5. The data demonstrates that higher concentrations of CTE (1% and 1.5%) tend to preserve total motility (TM), progressive motility (PM), and velocity parameters (e.g., VCL, VAP, and VSL) over prolonged incubation periods, compared to lower concentrations (0% and 0.5%). These findings suggest that CTE supplementation provides a protective effect against motility loss and oxidative stress during incubation, highlighting its potential in improving the post-thaw quality and functionality of cryopreserved semen.

DISCUSSION

The process of freezing and thawing semen is a stage that triggers oxidative stress, membrane damage, and decreased sperm motility quality, thus having a significant impact on fertilization potential (Türk et al., 2022). In this study, the addition of CTE significantly affected the main parameters of post-thawed semen quality, including viability, motility, plasma membrane integrity, acrosome integrity and abnormality. Sperm viability increased from 33.10% in control to 48.46% in the treatment with 1.5% CTE concentration. This increase reflects the ability of antioxidants in CTE, such as flavonoids and anthocyanins, to protect sperm cells from free radical damage during cryopreservation (Jamalan et al., 2016; Zhao et al., 2023). In addition, total motility showed significant improvement, with an increase from 50.85% (control) to 68.92% at 1% CTE concentration, although there was a slight decrease to 64.71% at 1.5% concentration. This suggests a potential mild toxic effect from accumulating active compounds at higher concentrations.

Kinematic parameters, such as progressive motility (PM) and curvilinear velocity (VCL), significantly improved with CTE treatment. Progressive motility reached 49.02% at 1.5% concentration, compared to 33.55% in the control. Meanwhile, VCL in CTE treatment remained more stable than in the control, indicating the ability of this treatment to maintain sperm metabolic energy essential for motility. The antioxidant ability of CTE in neutralizing free radicals seems to play an essential role in reducing mitochondrial damage, thereby improving sperm energy efficiency. This role is similar to that of flavonoids, particularly quercetin, and rutin, which at lower concentrations (20-30 μM) exhibit antioxidant properties that reduce lipid peroxidation and protect sperm from structural damage (El-Khawagah, Kandiel and Samir, 2020). Additionally, flavonoids can modulate energy pathways in sperm, affecting both glycolysis and mitochondrial respiration, which are crucial for motility (du Plessis et al., 2015; Yata et al., 2020).

Plasma membrane and acrosome integrity were also significantly improved by CTE treatment. At a concentration of 1.5%, membrane integrity increased to 53.74%, compared to 39.33% in control, while acrosome integrity reached 54.59% in the same treatment. This improvement suggests that the active compounds in CTE can protect the sperm membrane structure, which is highly susceptible to oxidative damage during thawing (Zribi et al., 2012; Baňas et al., 2023; Baňas et al., 2024; Partyka et al., 2024). Interestingly, sperm morphological abnormality parameters remained stable across all treatments, ranging from 14.72% to 15.26%, as shown in Table 2, indicating that CTE does not affect the morphological aspects but instead focuses more on the functional protection of the sperm, which is in contrast to the study by Swami et al. (Swami et al., 2017) that showed cysteamine, at a concentration of 5 mM, increased sperm morphological abnormalities in buffalo semen. With the ability to improve the viability, motility, and integrity of post-thawed sperm, bay flower extract offers an environmentally friendly solution as an alternative to commonly used synthetic antioxidants. The ability of CTE to protect sperm functional parameters without affecting morphology also adds to the advantages of its use in cryopreservation (Patel et al., 2016).

Semen storage at cold temperatures is a commonly used method in artificial insemination to maintain sperm quality (Karja et al., 2013; Galarza et al., 2019). However, oxidative stress during storage often leads to decreased motility and other kinematic parameters (Shaliutina-Kolešová & Nian, 2022). Based on this study's results, adding CTE to semen significantly reduced these adverse effects. Trajectory velocity parameters, such as curvilinear velocity (VCL), showed better stability in CTE treatment, especially at 1.5% concentration. On day 9, the VCL in the 1.5% group reached 87.72 μm/s compared to the control, which only reached 71.97 μm/s (Pilane & Mapeka, 2021). This increase indicates that the antioxidant compounds in CTE, such as flavonoids and anthocyanins, can protect sperm mitochondria from oxidative stress damage (Tiwari et al., 2022). In addition, total motility showed a slower decline with 1.5% CTE concentration, as shown in Figure 1. Furthermore, Figure 2 illustrates that on day 9, the 1.5% CTE treatment group exhibited the highest progressive motility (PM) value compared to the control, reflecting the protective effect of CTE in maintaining sperm metabolic energy during storage. (Yoshimoto et al., 2017; Khozein et al., 2024). Path average velocity (VAP) and straight line velocity (VSL) also showed better stability in the treatment group, indicating that CTE helped maintain sperm metabolic activity. On the other hand, sperm movement patterns measured through parameters such as linearity (LIN), straightness (STR), and wobble (WOB) showed no significant changes between treatments and controls. This suggests that CTE does not alter the basic pattern of sperm movement but instead focuses on protecting the structural integrity and metabolic activity of sperm during storage (Silvestre et al., 2021). Overall, these findings suggest that the addition of CTE may slow down the deterioration of semen quality during cold storage, increasing the chances of

successful fertilization in artificial insemination, especially in the context of semen transportation in remote areas. Sperm incubation after thawing simulates the physiological conditions encountered during fertilization, including exposure to thermal stress at body temperature (38°C) (Botta et al., 2019). In this study, treatment with CTE was shown to have a significant protective effect on semen quality, especially in the first 60 to 180 minutes of incubation. Total motility (TM) and progressive motility (PM) parameters showed significant improvement in the CTE-treated group compared to the control. At 60 minutes of incubation, a CTE concentration of 1.5% achieved TM values of 63.33% and PM of 46.38%, much higher than the control, which only reached 48.52% and 31.94%. This indicates the ability of active compounds in CTE, such as flavonoids and anthocyanin's, to protect sperm metabolism from oxidative damage in the early stages of incubation. However, at 300 minutes, all motility parameters decreased in all groups, including the CTE treatment (Cirit et al., 2013). Nonetheless, the 1.5% CTE group maintained higher TM and PM than the control, at 48.40% and 30.69%, respectively, while the control only reached 30.52% and 11.80%. Path velocity parameters such as curvilinear velocity (VCL), average path velocity (VAP), and straight-line velocity (VSL) showed similar trends, with the CTE treatment maintaining better velocity stability than the control. At 60 minutes of incubation, the VCL at 1% CTE concentration reached 78.48 µm/s compared to 70.95 µm/s for the control. The decrease after 300 minutes remained lower in the treatment group, with the VCL at 1.5% reaching 64.28 µm/s compared to the control, which only reached 50.65 µm/s (Díaz et al., 2024). Interestingly, the trajectory stability parameters (linearity, straightness, and wobble) showed no significant difference between treatment and control during incubation. This suggests that the addition of CTE does not structurally affect sperm movement patterns but instead focuses more on maintaining metabolism and preventing membrane damage due to oxidative stress (Agarwal et al., 2005). These results suggest that CTE can extend the functional time of sperm during early incubation, which is a critical period for fertilization. However, the deterioration after 300 min indicates the limit of CTE antioxidant protection against prolonged thermal damage. This study has significant practical implications for artificial insemination technology, particularly in the context of semen transportation in remote areas or farms with limited storage facilities. The addition of CTE in semen extenders may slow the deterioration of sperm quality during cold storage or thawing, increasing the chances of successful fertilization. In addition, bay flower extract as a natural antioxidant offers a more environmentally friendly and economical solution than synthetic antioxidants, such as BHT or vitamin E (Abdillah et al., 2021). With these findings, CTE-based semen extender formulations have the potential to be developed into commercial products for the livestock industry. Further research is needed to evaluate the application of CTE in other species, such as cattle, sheep, and even humans, and to find out if these compounds have similar effects on different types of sperm. In addition, further molecular studies are needed to understand the mechanism of interaction of CTE compounds with sperm components, such as membrane phospholipids and metabolic enzymes. Research could also be conducted to test the combination of CTE with other antioxidants, both natural and synthetic, to explore possible synergistic effects that could enhance protection against oxidative stress. This study uniquely contributes to developing semen cryopreservation technology, especially by introducing telling flower extract as a natural antioxidant. The uniqueness of this study lies in the first application of CTE in the treatment of Saanen goat semen, which showed significant results in improving sperm quality during cold storage and thawing. In addition, this study provides new insights into how natural antioxidants can replace more expensive and potentially harmful synthetic antioxidants while providing an environmentally friendly solution for the livestock industry.

***Conclusion*:** *Clitoria ternatea* extract significantly improved semen quality post-thaw, enhancing parameters such as viability, motility, and the integrity of the plasma membrane and acrosome. The antioxidant compounds in CTE, including flavonoids and anthocyanins, effectively protect spermatozoa from oxidative damage, improving kinematic performance and maintaining metabolic stability during cold storage and incubation. Notably, CTE slowed the deterioration of semen quality over time, especially in the early stages of incubation, providing a protective effect against oxidative stress. These findings suggest that CTE could be a natural, environmentally friendly alternative to synthetic antioxidants commonly used in semen cryopreservation. Further research is needed to explore its potential in other species and elucidate how its compounds interact with sperm components.

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A. Fajar: Compiling and designing experiments, conducting field experiments, and analyzing data.

***Ethical statement*:** This study has been approved by the Ethics Committee on the Use of Research and Learning Animals, Faculty of Animal Science, Hasanuddin University (No.001/UN4.12/EC/II/2025).

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***SDGs addressed*:** Zero Hunger; Good Health and Well-being; Industry, Innovation and Infrastructure

***Policy referred*:** Global Plan of Action for Animal Genetic Resources (FAO, 2007); Convention on Biological Diversity (CBD)

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